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I, KIM MARSHALL , MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed are true copies of the Provisional specification in connection with Application No. PO 8242 for a patent by MACQUARIE RESEARCH LTD, AUSTRALIAN WATER TECHNOLOGIES PTY LTD and BECTON DICKINSON AND COMPANY filed on 25 July 1997.

I further certify that the annexed specification is not, as yet, open to public inspection.

WITNESS my hand this Twenty-seventh day of May 1998

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Patents Act 1990

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BECTON DICKINSON AND COMPANY

PROVISIONAL SPECIFICATION

Invention Title:

Antibodies to Cryptosporidium

The invention is described in the following statement:

Technical Field

The present invention relates to antibodies to *Cryptosporidium* and methods to raise suitable *Cryptosporidium*-specific antibodies in animals.

Background Art

The protozoan parasite *Cryptosporidium* is amongst the most common pathogens responsible for diarrhoeal disease in humans. Infection occurs when *Cryptosporidium* oocysts shed in the faeces of infected individuals are ingested by new hosts. Recently, several large outbreaks of cryptosporidiosis have occurred in which drinking water has been identified as the source of infection. Surveys have shown that many surface water supplies are contaminated with *Cryptosporidium* oocysts.

Laboratory methods used to detect *Cryptosporidium* often involve the use of antibodies to this organism. Typical methods used to analyse water samples for the presence of this organism include microscopy and cytometry or a combination of these techniques. Flow cytometric methods involve staining of samples with a fluorescently labelled monoclonal antibody specific to the surface of *Cryptosporidium* oocysts and then analysis with a sorter flow cytometer. Particles with the fluorescence and light scatter characteristics of *Cryptosporidium* oocysts are sorted onto a microscope slide and examined manually using epifluorescence microscopy to confirm their identity as oocysts. This confirmation step is necessary because with a single antibody the cytometer is unable to distinguish oocysts from all other particles present in water samples. The particles that the cytometer can mistake as oocysts are autofluorescent particles such as algae or particles that non-specifically bind the oocyst-specific antibody.

Analysis-only flow cytometers are available which are simple to operate and relatively inexpensive. These cytometers are unable to perform sorting. To enable the detection of *Cryptosporidium* oocysts using an analysis-only cytometer the discrimination achieved by the cytometer must be improved so that non-oocyst particles are not mistaken as oocysts. The present inventors have shown previously that it is possible to detect a single specific microorganism in turbid water samples with an analysis cytometer if the microorganism is labelled with two different antibodies.

Unfortunately, the antibodies for *Cryptosporidium* presently available are not ideal due their stickiness and there is a need for more specific and reactive antibodies to the surface of *Cryptosporidium* oocysts. Monoclonal

antibodies (mAbs) that are specific to the surface of *Cryptosporidium* oocysts are used for detecting *Cryptosporidium* in clinical and environmental samples. All available mAbs that bind to the surface of *Cryptosporidium* oocysts are of the immunoglobulin M (IgM) or IgG3 subclass. Monoclonal antibodies of the IgG1 or IgG2 subclass would be preferable because they usually show less non-specific binding. Such mAbs would be more suitable in methods currently used for the detection and identification of *Cryptosporidium*. Unfortunately, past attempts by workers in the field to produce IgG1 or IgG2 monoclonal antibodies to *Cryptosporidium* have been unsuccessful or not substantiated (Smith, 1994; MacDonald *et al.*, 1991). It is generally considered that due to the antigenic characteristics of this organism, this class of antibody is not produced by infected or immunised animals (Smith, 1994).

In WO 97/08204 by the present inventors, monoclonal antibodies to a range of *Cryptosporidium* oocyst antigens were developed. Whole or excysted oocysts that were exposed to various treatments were used as antigens. From a total of 8 fusions that included screening several thousand hybridomas only one hybridoma was identified that was specific to the surface of *Cryptosporidium* oocysts. This monoclonal antibody was of the IgM immunological subclass.

The present inventors have now developed a new method that allows the production of IgG1 or IgG2 antibodies to the surface of *Cryptosporidium* oocysts.

Disclosure of Invention

In a first aspect, the present invention consists in a method of producing IgG1 or IgG2 subclass antibodies reactive to the surface of *Cryptosporidium* oocysts, the method comprising:

(a) separating at least a portion of the *Cryptosporidium* oocyst wall from the internal sporozoites to form an oocyst-wall preparation;

(b) treating the separated oocyst-wall preparation so as to obtain an oocyst antigen preparation capable of eliciting a detectable IgG1 or IgG2 immune response in an animal to the surface of the oocyst;

(c) immunising an animal with the oocyst antigen preparation so as to elicit an IgG1 or IgG2 immune response in the animal; and

(d) obtaining IgG1 or IgG2 antibodies reactive to the surface of *Cryptosporidium* oocysts from the animal.

The present inventors have found that in order to obtain a suitable oocyst wall antigen preparation, the oocyst wall should be separated from internal sporozoite components. It appears that the internal sporozoite antigens are more immunodominat than oocyst wall antigens and their presence in an antigen preparation may mask the oocyst wall antigens. A mixed antigen preparation will usually result in raising antibodies to the sporozoite antigens.

The separation of the oocyst wall from the internal sporozoite (step (a)) can be achieved by any means. The present inventors have found that causing the oocyst to excyst followed by immuno-separation of the wall components is particularly suitable. It will be appreciated, however, that other separation methods known to the art would also be suitable. Examples include centrifugation, flow cytometry, density gradient separation, precipitation, and chromatographic separation.

It is not necessary to cause the oocyst to excyst by normal procedures. The oocysts can be freeze-thawed for example to promote initial separation of the wall from the internal sporozoites. Furthermore, the oocyst may be physically broken up by crushing, sonication, or grinding followed by separation.

The treating step (b) can be carried out by any means suitable. In particular, the present inventors have found that physically breaking up the cell wall can produce a good antigen preparation. This can be done by any means but the use of a bead beater is quite suitable.

The treatment removes antigens from the surface of the oocyst wall in a form that will allow the generation of IgG1 or IgG2 antibodies when injected into an animal.

It will be appreciated that once a suitable immune response has been stimulated in an animal, for example in a laboratory mouse, monoclonal antibodies of IgG1 or IgG2 subclass may be generated by standard techniques from that animal.

The animal may be immunised by any technique suitable for eliciting an immune response in an animal. Adjuvants may also be included with the antigen preparation prior to immunising the animal to promote a strong immune response in the animal.

In a further preferred embodiment, the antigen preparation also enhances the production of IgM antibodies when placed in an animal.

In a second aspect, the present invention consists in substantially isolated IgG1 or IgG2 antibodies reactive to the surface of *Cryptosporidium* oocysts produced by the method according to the first aspect of the present invention.

Preferably, the antibodies are monoclonal antibodies.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples.

Modes for Carrying Out the Invention

MATERIALS AND METHODS

***Cryptosporidium* oocysts**

Cryptosporidium parvum oocysts were purified from pooled faeces of naturally infected neonatal calves in Sydney. Faecal samples were centrifuged (2000 g, 10 min) and resuspended in water twice and then resuspended in 5 volumes of 1% (w/v) NaHCO3. Fatty substances were then extracted twice with 1 volume of ether, followed by centrifugation (2000 g for 10 min). Pellets were resuspended in water and filtered through a layer of pre-wetted non-adsorbent cotton wool. The eluate was then overlaid onto 10 volumes of 55% (w/v) sucrose solution and centrifuged (2000 g for 20 min). Oocysts were collected from the sucrose interface and the sucrose flotation step repeated until no visible contaminating material could be detected. Purified oocysts were surface sterilised with ice cold 70% (v/v) ethanol for 30 min, washed once in phosphate buffered saline (PBS; Oxoid, Sydney) and stored in PBS at 4^0C for up to 2 weeks.

Antigen preparation

Excystation of oocysts

A 1 ml sample containing approximately 5×10^9 oocysts was excysted as described by Robertson *et al.* (1993). The sample was centrifuged at 13,000 g for 1 minute and the supernatant removed and discarded. The pellet was resuspended in 1 ml of Hank's balanced salt solution (HBSS), pH 2.7, and incubated at 37°C for 30 min. The sample was then washed and resuspended in 100 µl of PBS with 10 µl of 1% (w/v) sodium deoxycholate in Hank's minimal essential medium (HMEM) and 10 µl of 2.2% (w/v) $NaHCO_3$ in HBSS, and incubated at 37°C for 4 h.

Purification of oocyst walls

The oocysts walls were purified from the excysted sample using immuno-magnetic separation. A 0.5 ml aliquot of magnet beads

(approximately 5 x 10^7 beads) coated with a goat anti-mouse IgM antibody (Dynal Pty Ltd, Australia) were mixed with 10 ml of tissue culture supernatant of a *Cryptosporidium* oocyst-specific monoclonal antibody CRY26 (Vesey 1996). The beads were incubated at 4°C for 4 hours and then placed next to a magnet so that the beads were drawn to the bottom of the tube. The supernatant was removed and discarded and the beads resuspended in 10 ml of PBS plus 2% (w/v) bovine serum albumin (BSA; Sigma fraction V)(PBS-BSA). This washing procedure was repeated twice and the beads resuspended in a final volume of 1 ml of PBS-BSA. The beads were then mixed with the sample of excysted oocysts and incubated on a rotary shaker at room temperature for 30 minutes. The tube was placed next to the magnet so that the beads and the attached oocysts were attracted to the bottom of the tube. The supernatant was removed and placed at 4°C. To remove any contaminating sporozoites the beads were gently resuspended in 1 ml of PBS-BSA and then concentrated once more using the magnet. The supernatant was removed and discarded. The beads were resuspended in 1 ml of PBS and vortexed vigorously to unattached the beads from the oocyst walls. The beads were concentrated using the magnet and the supernatant containing the oocyst walls removed and kept on ice. The beads were then added to the original sample of excysted oocysts and the entire procedure repeated 10 times. The 10 samples of purified oocyst walls were then pooled and concentrated by centrifuging at 3000 g for 10 minutes. A fraction of the sample of oocyst walls was analysed using flow cytometry as described previously (Vesey et al., 1997). The sample was analysed in a tube containing an exact number of beads (TrueCount, Becton Dickinson, San Hose, USA) to determine the number of oocyst walls.

Breaking up the oocysts walls

Half the sample of oocyst walls were treated to break the walls into small pieces using a FastPrep bead beater (Bio101, CA, USA) fifteen times at maximum speed for 40 second durations. The sample was cooled on ice for 1 minute between each 40 second treatment. The oocyst wall pieces were resuspended into 3 ml of PBS and aliquoted into 200 µl amounts and stored frozen until used.

Immunisation of mice

Five Balb/C female mice (group WP) were immunised by IP injection with 200 µl of smashed oocyst wall preparation emulsified in Freunds

complete adjuvant. The preparation contained approximately 1 x 10^6 oocyst walls. A second group of mice (group W) were immunised with approximately 1 x 10^6 intact purified oocyst walls emulsified in Freunds complete adjuvant. Mice were bled by tail bleeding prior to receiving the primary injection to provide a negative control. Two further IP injections with the same amount of antigen but emulsified in Freunds incomplete adjuvant were given at 3 week intervals. Mice were bled after the second of these injections to check for immune response.

The mouse may be given either IP or IV booster injections to 7 days prior to sacrifice and fusion of spleen cells to assist in the development of appropriate antibodies.

Analysis of mouse serum

Samples (approximately 50 µl) of blood were collected by tail bleeding and then centrifuged at 13000 g for 30 seconds. The top layer of serum was carefully removed and stored at -20°C until analysed. Serum was diluted to 1 in 100, 1 in 1000 and 1 in 10,000, in PBS-BSA. Aliquots (50 µl) of diluted serum were mixed with 10 µl of oocyst suspension in PBS (containing approximately 1 x 10^6 oocysts) and incubated at room temperature for 20 minutes. Samples were mixed with either 50 µl of goat anti-mouse IgM specific antibody conjugated with FITC (diluted 1 in 50 with BSA-PBS) (either Sigma product number F9254 or Sigma) or with 50 µl of goat anti-mouse IgG specific antibody conjugated with either PE (diluted 1 in 200 with BSA-PBS)(Sigma P0188) or conjugated with FITC (diluted 1 in 200 with BSA-PBS) (Zymed Laboratories Inc., San Francisco, USA). After a further 20 minutes incubation at room temperature samples were analysed using a FACScan flow cytometer. A negative control of PBS and an IgM positive control of tissue culture supernatant from a IgM monoclonal antibody specific to the surface of *Cryptosporidium* oocysts were analysed with each batch of samples. The mean fluorescence intensities of the FITC and the PE stained samples were recorded.

Samples of mouse serum diluted 1 in 500 in BSA-PBS were analysed using western blotting.

Flow cytometry

A FACScan flow cytometer was used for analysis of mouse serum and hybridomas. Logarithmic signals were used for all detectors. The threshold was set on side scatter at a value of 500. The detectors were set at the

following levels of sensitivity: 200 for side scatter (SSC); E00 for forward scatter (FALS); 600 for the green fluorescence detector (FL1) and 600 for the red fluorescence detector (FL2). A region (R1) was defined on a dot plot of FALS versus SSC that enclosed single oocysts but not clumps of oocysts. Histograms of FL1 and FL2 were gated so that the only particles that appeared in region R1 would appear on the histograms. The mean value of FL1 and or FL2 from the histograms were recorded for 2000 oocysts from each sample analysed.

ALTERNATIVE METHODS

To separate the oocyst walls from the sporozoites the oocysts can be freeze-thawed instead of excysted. A purification step such as immuni-magnetic separation, flow cytometry or density gradient separation is required to purify the oocyst walls away from the sporozoites.

One alternative approach would involve smashing the whole oocysts (sporozoites still inside) into small pieces and then using an immunological purification method such as immuno-magnetic separation or affinity chromatography.

Other alternative methods such as sonication could be used to break up the oocysts or the purified oocyst walls.

RESULTS

Flow cytometric analysis of serum from mice immunised with intact purified oocyst walls (group W mice) revealed no difference in the brightness of *Cryptosporidium* oocysts stained with serum collected before or after immunisation (Table 1). Similar results were observed when using both IgM specific and IgG specific second antibodies. In comparison, when oocysts were stained with the serum from group WP mice there was a difference in the fluorescence intensity of the oocysts stained with post immunisation serum and those stained with pre-immunisation serum. Results were similar for oocysts stained with both IgG specific and IgM specific secondary antibodies.

Table 1. Comparison of the fluorescence intensity of *Cryptosporidium* oocysts stained with serum (diluted 1 in 100 or 1 in 1000) from group W and group WP mice and then stained with an anti-IgG or an anti-IgM fluorescently labelled antibody. Serum was tested prior to immunisation and then 21 days after the second immunisation.

	IgG specific antibody		IgM specific antibody	
mouse number	Pre-immunisation	Post-immunisation	Pre-immunisation	Post-immunisation
[1]W1	36	39	16	17
W2	45	32	27	14
W3	36	37	27	13
W4	42	30	25	27
W5	47	40	16	15
[2]WP1	[3]230	248	404	420
WP2	231	**518**	405	**514**
WP3	229	**306**	403	**475**
WP4	233	**290**	410	417
WP5	232	**350**	407	**603**

[1]W - mice immunised with intact purified oocyst walls. Serum was diluted 1 in 1000.

[2]WP - mice immunised with small pieces of purified oocyst walls. Serum was diluted 1 in 100.

[3]Results of the two groups of mice are not directly comparable. Serum was not tested at the same dilution for both groups of mice. Different second antibodies were used to test the group W mice and the group WP mice.

CONCLUSIONS

Mice immunised with purified *Cryptosporidium* oocysts walls do not produce a strong immunological response to the surface of *Cryptosporidium* oocysts. However, if the purified oocysts walls are broken up into small pieces then a strong response is produced in both the IgG and IgM immunological subclasses. Mice immunised with such a procedure would be suitable for producing monoclonal antibodies of the IgG1 or IgG2 subclasses.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty-fifth day of July 1997

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REFERENCES

Robertson L. J., Campbell A. T. & Smith H. V. 1993. *In vitro* excystation of *Cryptosporidium parvum*. *Parasitology* **106**: 13-19.

Vesey G. 1996. Application of flow cytometry to the detection of *Cryptosporidium* in water. PhD thesis, Macquarie University, Sydney, Australia.

Vesey, G, Griffiths, K, Gauci, M, Deere, D, Williams, K and Veal, D. 1997. Simple and rapid measurement of *Cryptosporidium* excystation using flow cytometry. *International Journal of Parasitology*, in press.

McDonald,V; Deer,RM; Nina,JM; Wright,S; Chiodini,PL; McAdam,KP. (1991). Characteristics and specificity of hybridoma antibodies against oocyst antigens of *Cryptosporidium parvum* from man. Parasite Immunology **13**, 251-259.

Smith, H (1994). Use of antibodies for detecting environmental contamination with protozoan parasites. In Laboratory Notes for Water Microbiology for the 21st Century, University of Nottingham, U.K., 21-23 September.

Vesey, G, Slade, JS, Byrne, M, Shepherd, K, Dennis, PJL, and Fricker, CR. (1993). A new method for the concentration of *Cryptosporidium* oocysts from water. Journal of Applied Bacteriology, **75**, 87-90.